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III. 研究成果の刊行物・別冊

The NS3 Helicase and NS5B-to-3'X Regions Are Important for Efficient Hepatitis C Virus Strain JFH-1 Replication in Huh7 Cells[∇]

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The JFH-1 strain of hepatitis C virus (HCV) is a genotype 2a strain that can replicate autonomously in Huh7 cells. The J6 strain is also a genotype 2a strain, but its full genomic RNA does not replicate in Huh7 cells. However, chimeric J6/JFH-1 RNA that has J6 structural-protein-coding regions and JFH-1 nonstructural-protein-coding regions can replicate autonomously and produce infectious HCV particles. In order to determine the mechanisms underlying JFH-1 RNA replication, we constructed various J6/JFH-1 chimeras and tested their RNA replication and virus particle production abilities in Huh7 cells. Via subgenomic-RNA-replication assays, we found that both the JFH-1 NS5B-to-3'X (NSBX) and the NS3 helicase (N3H) regions are important for the replication of the J6CF replicon. We applied these results to full-length genomic RNA replication and analyzed replication using Northern blotting. We found that a chimeric J6 clone with JFH-1 N3H and NSBX could replicate autonomously but that a chimeric J6 clone with only JFH-1 NSBX had no replication ability. Finally, we tested the virus production abilities of these clones and found that a chimeric J6 clone with JFH-1 N3H and NSBX could produce infectious HCV particles. In conclusion, the JFH-1 NS3 helicase and NS5B-to-3'X regions are important for efficient replication and virus particle formation of HCV genotype 2a strains.

Hepatitis C virus (HCV) is a major cause of chronic liver disease (7, 22). The lack of a robust cell culture system for producing virus particles has hampered the development of HCV research (2). Although the development of a subgenomic-replicon system enabled research into HCV RNA replication (32), infectious-virus-particle production remained impossible. Recently, an HCV cell culture system was developed using a JFH-1 genotype 2a strain of HCV cloned from a fulminant hepatitis patient (30, 48, 54), allowing investigation of the virus life cycle.

HCV is a positive-strand RNA virus that belongs to the *Hepacivirus* genus in the *Flaviviridae* family. The HCV genome comprises about 9,600 nucleotides that encode a single polyprotein of around 3,000 amino acids (8, 18, 44), which is processed by cellular and viral encoded proteases into at least 10 different structural and nonstructural proteins (11, 13, 14, 33).

The JFH-1 strain of HCV is a genotype 2a strain, and it is the first HCV strain that can produce HCV particles in Huh7 cells (48). Subgenomic replicons of JFH-1 replicate efficiently in Huh7 cells and do not require cell culture-adaptive mutations (19). The J6CF strain of HCV is also a genotype 2a strain and is known to be infectious in chimpanzees (49), but its

entire genomic RNA does not replicate in Huh7 cells, despite the ~90% nucleotide sequence homology between JFH-1 and J6CF. However, J6/JFH-1 chimeric RNA that has J6 structural-protein-coding regions and JFH-1 nonstructural-protein-coding regions can replicate autonomously and produce infectious HCV particles (30, 39). Why only the JFH-1 clone can replicate efficiently in Huh7 cells remains unclear.

In this study, to investigate the mechanisms underlying efficient JFH-1 replication, we focused on the differences in replication between JFH-1 and J6CF strains by using intragenotypic JFH-1 and J6CF chimeras and compared their respective abilities to replicate RNA and produce virus particles in Huh7 cells.

MATERIALS AND METHODS

Cell culture. Huh7 cells (36) were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum under 5% CO₂ conditions.

Subgenomic-replicon constructs. pSGR-JCH1 and pSGR-JCH4 were constructed based on pSGR-JFH1 (19, 21). pSGR-J6CF was also constructed from pJ6CF (a kind gift from Jens Bukh) (49), using the same method used to construct pSGR-JFH1. Plasmids used in luciferase assays were constructed based on pSGR-JFH1/Luc (20). Chimeric replicons were constructed by substitution of the corresponding regions. For convenience, several restriction enzyme recognition sites (ClaI [2275], EcoT22I [3639], and BsrGI [6127]) were introduced into the pSGR-J6CF sequence via nucleotide substitutions. The substitutions of the corresponding regions were achieved as follows, with the 5' untranslated region (5' UTR) inserted between NotI and AgeI: NS3, PmeI-EcoT22I; NS3 protease, PmeI-ClaI; NS3 helicase, ClaI-EcoT22I; NS4, EcoT22I-MunI; NS5A, MunI-BsrGI; NS5B, BsrGI-StuI; and 3' UTR, StuI-XbaI (see Fig. 2A and 3A). pSGR-JCH1/Luc and pSGR-JCH4/Luc were also constructed using the same procedure as that for pSGR-JFH1/Luc (20, 21). The Con1 replicon (pSGR-Con1/Luc) was

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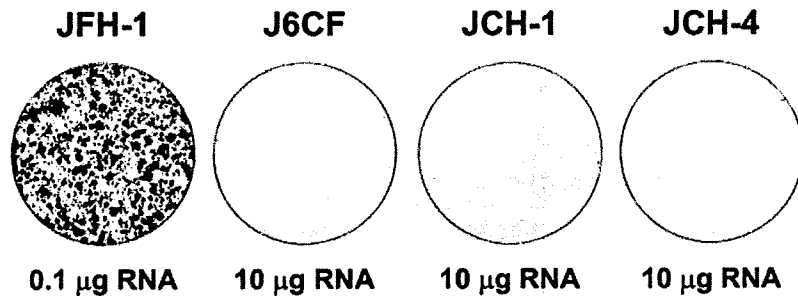


FIG. 1. G418-resistant colony formation of JFH-1, J6CF, JCH-1, and JCH-4. Subgenomic RNAs were synthesized *in vitro*, using pSGR-JFH1, pSGR-J6CF, pSGR-JCH1, and pSGR-JCH4 as templates. Transcribed subgenomic RNAs were electroporated into Huh7 cells, and cells were cultured with G418 for 3 weeks before staining with crystal violet as described in Materials and Methods. JFH-1 subgenomic RNA (0.1 μ g) and 10 μ g of J6CF, JCH-1, and JCH-4 subgenomic RNAs were transfected into Huh7 cells. Experiments were performed in triplicate, and representative staining examples are shown.

constructed from pFK-I389/neo/NS3-3'/wt (a kind gift from Ralf Bartenschlager) (32), and the H77c replicon (pSGR-H77c/Luc) was constructed from pCV-H77c (a kind gift from Robert H. Purcell) (50). For convenience, ClaI (2275) and BsrGI (6127) recognition sites were introduced into the pSGR-Con1/Luc and pSGR-H77c/Luc sequences via nucleotide substitutions. Substitutions of the NS3 helicase region and NS5B regions were performed as described above.

Full-length genomic HCV constructs. Plasmids used in the analysis of genomic RNA replication were constructed based on pJFH1 (48) and pJ6CF (49). For convenience, several restriction enzyme recognition sites (ClaI [3929], EcoT22I [5293], and BsrGI [7781]) were introduced into the J6CF sequence via nucleotide substitutions. Substitutions of the NS3 helicase regions were performed by replacement of the ClaI-EcoT22I fragment, substitutions of the NS5B regions were performed by replacement of the BsrGI-XbaI fragment, substitutions of the NS5A regions were performed by replacement of the BsrGI-StuI fragment, and a substitution of the 3'UTR was performed by replacement of the StuI-XbaI fragment (see Fig. 5A).

RNA synthesis and transfection. RNA synthesis and transfection were performed as described previously (48). In brief, plasmids were linearized with XbaI, treated with mung bean nuclease (New England Biolabs, Ipswich, MA), and purified. Linearized, purified DNAs were used as templates for *in vitro* RNA synthesis using a MEGAscript T7 kit (Ambion, Austin, TX) in accordance with the manufacturer's instructions. Synthesized RNA was treated with DNase I (Ambion), followed by purification using ISOGEN-LS (Nippon Gene, Tokyo, Japan). The quality of synthesized RNA was examined by agarose gel electrophoresis. Ten micrograms of *in vitro*-synthesized RNA was used for each electroporation. Trypsinized Huh7 cells (3×10^6 cells) were washed with Opti-MEM I (Invitrogen, Carlsbad, CA) and resuspended in Cytomix buffer (47). RNA was mixed with 400 μ l of cell suspension, and the mixture was then transferred to an electroporation cuvette (Precision Universal Cuvettes, Thermo Hybaid, Middlesex, United Kingdom). The cells were then pulsed at 260 V and 950 μ F using a Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells were immediately transferred to 10-cm culture dishes or six-well plates, each containing culture medium, and incubated at 37°C under 5% CO₂. Luciferase mRNA was synthesized from luciferase T7 control DNA (Promega, Madison, WI) by using a mMMESSAGE mMACHINE T7 kit (Ambion). To monitor transfection efficiency, *in vitro*-synthesized luciferase RNA was cotransfected with HCV RNA and luciferase activity measured at 4 h after transfection.

G418-resistant colony formation assay. The G418-resistant colony formation assay was performed as described previously (19). In brief, 0.1 μ g or 10 μ g of transcribed RNAs was transfected into 3×10^6 Huh7 cells by electroporation. Transfected cells were immediately transferred to 10-cm culture dishes containing 10 ml of culture medium. G418 (1.0 mg/ml) (Nakalai Tesque, Kyoto, Japan) was added to the culture medium at 16 to 24 h after transfection. Culture medium supplemented with G418 was replaced every 3 days. Three weeks after transfection, cells were fixed with buffered formalin and stained with crystal violet.

Luciferase reporter assay. The luciferase activities of the JFH-1 subgenomic replicon and chimeras in Huh7 cells were measured as described previously (20). Briefly, 5 μ g of transcribed RNAs was transfected into 3×10^6 Huh7 cells by electroporation. Transfected cells were immediately resuspended in culture medium and seeded into six-well culture plates. Cells were harvested serially at 4, 24, and 48 h after transfection and lysed with 200 μ l of cell culture lysis reagent

(Promega). Debris was then removed by centrifugation. Luciferase activity was quantified using a Lumat LB9507 luminometer (EG & G Berthold, Bad Wildbad, Germany) and a luciferase assay system (Promega). Assays were performed three times independently, with each value corrected for transfection efficiency as determined by measuring luciferase activity 4 h after transfection. The data are expressed as relative luciferase units (RLU).

Quantification of HCV core protein. To estimate the concentration of HCV core protein in the culture medium, we performed an HCV core enzyme-linked immunosorbent assay (Ortho-Clinical Diagnostics, Tokyo, Japan) in accordance with the manufacturer's instructions.

Northern blot analysis. Northern blot analysis was performed as described previously (48). In brief, total cellular RNA from HCV RNA-transfected cells was extracted using ISOGEN (Nippon Gene) in accordance with the manufacturer's instructions. Isolated RNA (2 μ g) was separated on a 1% agarose gel containing formaldehyde, transferred to a Hybond N+ positively charged nylon membrane (GE Healthcare, Piscataway, NJ), and immobilized using a Stratilinker UV cross-linker (Stratagene, La Jolla, CA). Hybridization was performed with [α -³²P]dCTP-labeled DNA by using Rapid-Hyb buffer (GE Healthcare). The DNA probe was synthesized using the NSSB-to-3'X fragment of JFH1 excised from pJFH1 by BsrGI and XbaI and labeled using the Megaprime DNA labeling system (GE Healthcare).

Infection of cells with secreted HCV and determination of infectivity. Culture medium from RNA-transfected cells was collected at 72 h posttransfection. Huh7 cells were seeded at a density of 1×10^4 cells per well in poly-D-lysine-coated 96-well plates (CORNING, Corning, NY). On the following day, the collected culture media were serially diluted and used for inoculation of the seeded cells, and the plates were incubated for another 3 days at 37°C. The cells were fixed in methanol for 15 min at -20°C, and the infected foci were visualized by immunofluorescence as described below.

Cells were blocked for 1 h with BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) supplemented with 0.3% Triton X-100 and then washed with phosphate-buffered saline, followed by incubation with anti-core antibody at 50 μ g/ml in BlockAce. After incubation for 1 h at room temperature, the cells were washed and incubated with a 1:400 dilution of AlexaFluor 488-conjugated anti-mouse immunoglobulin G (Molecular Probes, Eugene, OR) in BlockAce. The cells were then washed and examined using fluorescence microscopy (Olympus, Tokyo, Japan). Infectivity was quantified by counting the infected foci and expressed as numbers of focus-forming units per milliliter (FFU/ml).

RESULTS

G418-resistant colony formation of JFH-1, J6CF, and other genotype 2a subgenomic replicons. First, to compare the replication efficiencies of the JFH-1 and J6CF strains, we performed a G418-resistant colony formation assay with JFH-1 and J6CF RNAs by using subgenomic replicons. The JFH-1 subgenomic replicon formed many colonies with transfection of only 0.1 μ g RNA, but the J6CF subgenomic replicon formed no colonies, even with transfection of 10 μ g RNA (Fig. 1). We also tested

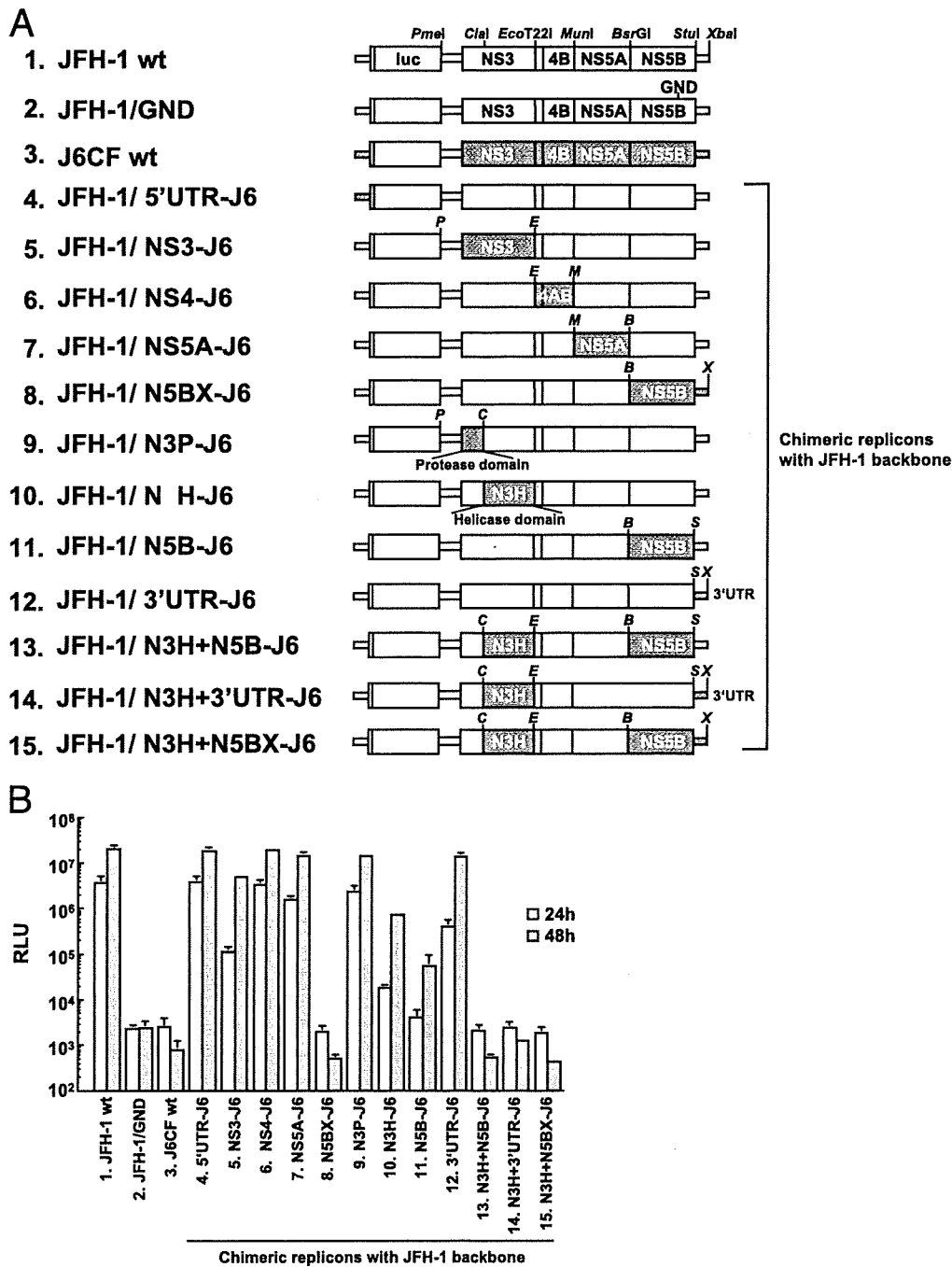


FIG. 2. Luciferase activities of chimeric replicons with a JFH-1 backbone. (A) Structures of chimeric subgenomic replicons with a JFH-1 backbone. The restriction enzyme recognition sites used for the construction of plasmids are indicated. *P*, *PmeI*; *C*, *ClaI*; *E*, *EcoT22I*; *M*, *MunI*; *B*, *BsrGI*; *S*, *StuI*; *X*, *XbaI*; wt, wild type. (B) Subgenomic RNAs were synthesized in vitro from wild-type or chimeric replicon constructs. Transcribed subgenomic RNAs (5 μ g) were electroporated into Huh7 cells, and cells were harvested serially at 4, 24, and 48 h after transfection. The harvested cells were lysed, and then luciferase activities in the cell lysates were measured. The assays were performed three times independently and the results expressed as luciferase activities (RLU). Each value was corrected for transfection efficiency as determined by measuring the luciferase activity 4 h after transfection. Data are presented as means and standard deviations for luciferase activity at 24 h (white bars) and 48 h (gray bars) after transfection.

other genotype 2a clones (the JCH-1 and JCH-4 strains), which were isolated from patients with chronic hepatitis C (21). Their subgenomic replicons did not form colonies either. Given that chimeric J6/JFH-1 RNA that has the J6 structural-protein-coding

regions and JFH-1 nonstructural-protein-coding regions reportedly replicates autonomously and produces infectious HCV particles (30, 39), we hypothesized that some of the JFH-1 nonstructural-protein-coding regions are important for JFH-1 replication.

Regions of JFH-1 essential for replication. In order to determine which regions of JFH-1 are important for JFH-1 RNA replication, we constructed a series of chimeric JFH-1 subgenomic replicons replacing the 5'UTR, NS3, NS4AB, NS5A, and NS5B-to-3'X (N5BX) regions from the J6CF strain and tested their replication abilities. For this analysis, we adopted luciferase replicon systems (20) because colony formation assays are time-consuming to perform and it is difficult to evaluate precise replication levels using this method. Furthermore, efficient JFH-1 RNA replication may reduce cellular growth, thus affecting colony formation efficiency (34). We constructed JFH-1 chimeric subgenomic luciferase replicons with the J6CF clone because this clone was reportedly infectious in a chimpanzee (49). However, the JCH-1 and JCH-4 clones were not tested for infectivity. The 5'UTR, NS3, NS4AB, NS5A, or N5BX sequences of the JFH-1 replicon were replaced by J6CF sequences (5'UTR-J6, NS3-J6, NS4-J6, NS5A-J6, or N5BX-J6, respectively [Fig. 2A]). The luciferase activities of these replicons are shown in Fig. 2B. The JFH-1 subgenomic replicon replicated efficiently and had a luciferase activity of approximately 10^7 RLU (Fig. 2B, JFH-1 wt). GND, which was replication incompetent because of a mutation at the GDD motif in the NS5B region, had a luciferase activity of only 10^3 RLU (Fig. 2B, JFH-1/GND), which was taken as the background level. The J6CF subgenomic replicon did not replicate and had the same luciferase activity as GND (Fig. 2B, J6CF wt). Replacement of the 5'UTR, NS4AB, and NS5A sequences of JFH-1 by J6CF sequences (5'UTR-J6, NS4-J6, and NS5A-J6, respectively) did not reduce replication (Fig. 2B, 5'UTR-J6 and NS4-J6) or reduced it only slightly (Fig. 2B, NS5A-J6). However, there was no replication for the JFH-1 chimera with J6 N5BX (Fig. 2B, N5BX-J6). In addition, the JFH-1 chimera with the J6 NS3 region (NS3-J6) had a replication level that was more than 10-fold lower at 24 h and around 10-fold lower at 48 h than that of the wild-type JFH-1 replicon (Fig. 2B, JFH-1 wt and NS3-J6). These data show that the JFH-1 NS5B-to-3'X region is essential for JFH-1 RNA replication and indicate that the JFH-1 NS3 region is also important for JFH-1 RNA replication.

Involvement of the NS3 helicase region in efficient JFH-1 replication. The JFH-1 chimera with the J6 NS3 region (NS3-J6) reduced the replication level (Fig. 2B, NS3-J6). The NS3 protein is known to have two domains: a protease domain at the amino terminal one-third and a helicase domain at the carboxyl terminal two-thirds. To determine which region is important for replication, we compared the replication activity of a JFH-1 chimera with that of the NS3 protease-coding region of J6CF (N3P-J6) and that of a JFH-1 chimera with that of the NS3 helicase-coding region of J6CF (N3H-J6) (Fig. 2A, JFH-1/N3P-J6 and JFH-1/N3H-J6). Although N3P-J6 had the same luciferase activity as JFH-1, N3H-J6 had lower activity than JFH-1 (Fig. 2B, N3P-J6 and N3H-J6). These data show that the JFH-1 NS3 helicase-coding region has an important role in JFH-1 replication.

Importance of the JFH-1 NS5B-coding region and 3'UTR in replication. The JFH-1 chimera with J6 N5BX completely abolished replicon replication (Fig. 2B, N5BX-J6). The N5BX region contains two regions, the NS5B protein-coding region and the 3'UTR. The NS5B protein-coding region encodes RNA-dependent RNA polymerase. To analyze which region of

N5BX is important for replication, we separated N5BX into two regions, that is, the NS5B-coding region and the 3'UTR. JFH-1 replicons with NS5B or with the 3'UTR of J6 were constructed (Fig. 2A, JFH-1/NS5B-J6 and JFH-1/3'UTR-J6) and their replication abilities analyzed. The replication level of JFH-1/NS5B-J6 was reduced more than 100-fold compared with that of the wild-type JFH-1 replicon at 48 h (Fig. 2B, N5B-J6). JFH-1/3'UTR-J6 replicated similarly to JFH-1 at 48 h, but the replication activity at 24 h was reduced more than 10-fold compared with that of the original JFH-1 replicon (Fig. 2B, 3'UTR-J6). These data indicate that the NS5B-coding region and the 3'UTR of JFH-1 are both involved in efficient JFH-1 replication.

Rescue of J6CF replicon replication by incorporation of the JFH-1 sequences. Because the JFH-1 N5BX region appeared to be essential for JFH-1 replication (Fig. 2B, N5BX-J6), we tested whether JFH-1 N5BX could restore the replication of J6CF RNA. We constructed a chimeric J6CF subgenomic replicon containing the JFH-1 N5BX region (Fig. 3A, J6/N5BX-JFH1) and tested its replication abilities. The luciferase activity of J6CF subgenomic RNA was recovered by inclusion of JFH-1 N5BX (Fig. 3B, N5BX-JFH1), but this chimeric replicon showed lower replication activity than the original JFH-1 replicon (Fig. 3B, JFH-1 wt). Furthermore, J6CF replication was not restored by only JFH-1 NS5B (J6/N5B-JFH1) or only the 3'UTR (J6/3'UTR-JFH1) (Fig. 3B, N5B-JFH1 or 3'UTR-JFH1, respectively). These observations clearly indicate that the JFH-1 NS5B-to-3'X region is essential, and the NS5B-coding region and 3'UTR are both important for efficient RNA replication in Huh7 cells. However, other JFH-1 regions are also involved in efficient replication.

The JFH-1 NS3 helicase-coding region was also important for efficient replication, and we thus tested whether the JFH-1 NS3 helicase region by itself could restore J6CF replication (as occurred for the JFH-1 N5BX region). Insertion of only the NS3 helicase region of JFH-1 into J6CF (Fig. 3A, J6/N3H-JFH1) did not restore replication (Fig. 3B, N3H-JFH1). However, replication of the J6 chimeric replicon seemed considerably restored by insertion of JFH-1 NS5B or the 3'UTR in addition to the NS3 helicase-coding region (Fig. 3B, N3H+N5B-JFH1 or N3H+3'UTR-JFH1, respectively) and fully restored by insertion of the JFH-1 NS3 helicase region and JFH-1 N5BX region (Fig. 3B, N3H+N5BX-JFH1). These results indicate that the JFH-1 N5BX region is essential for subgenomic-replicon replication and that the JFH-1 NS3 helicase-coding region has an additional role in replication. This was also confirmed by analysis of the replication abilities of JFH-1 replicons with double substitutions of J6CF (Fig. 2A, JFH-1/N3H+N5B-J6, JFH-1/N3H+3'UTR-J6, and JFH-1/N3H+N5BX-J6). Neither of these chimeric JFH-1 replicons replicated (Fig. 2B, N3H+N5B-J6, N3H+3'UTR-J6, and N3H+N5BX-J6).

The NS3 helicase and NS5B-3'X regions of JFH-1 can restore the replication of other genotype 2a replicons but not of genotype 1 replicons. To test whether the JFH-1 NS3 helicase and N5BX regions could restore other HCV replicon replication, chimeric replicon constructs N3H-JFH1, N5BX-JFH1, and N3H+N5BX-JFH1 were constructed using two genotype 2a replicons (JCH-1 and JCH-4), a genotype 1a replicon (H77c), and a genotype 1b replicon (Con1), respectively. The

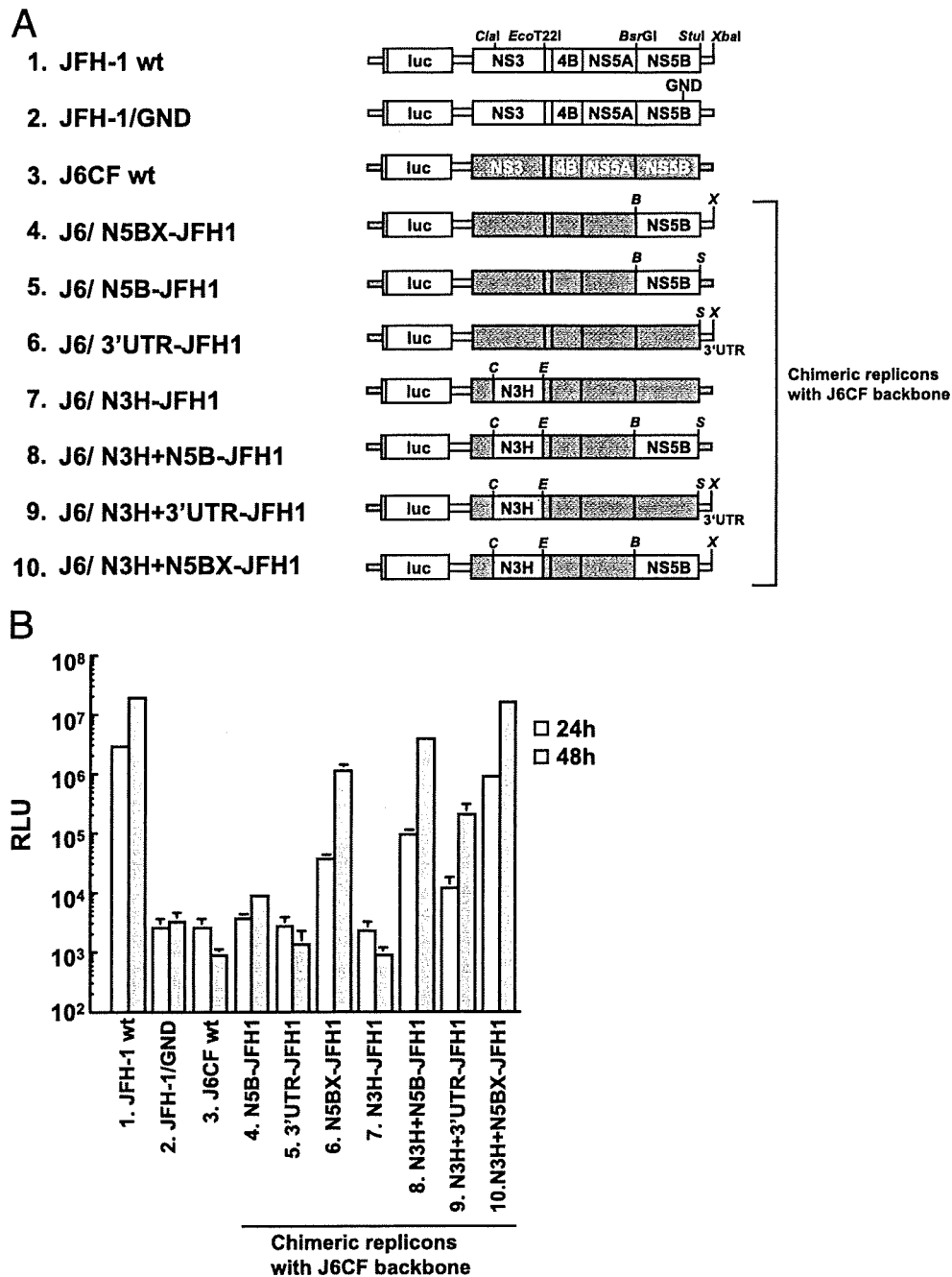


FIG. 3. Luciferase activities of chimeric replicons with a J6CF backbone. (A) Structures of chimeric subgenomic replicons with a J6CF backbone. The restriction enzyme recognition sites used for the construction of plasmids are indicated. C, Clal; E, EcoT221; B, BsrGI; S, StuI; X, XbaI; wt, wild type. (B) Wild-type or chimeric subgenomic RNAs were transfected into Huh7 cells, and the luciferase activities of the transfected cells were examined as described in the legend to Fig. 2B. Assays were performed three times independently, and data are presented as means and standard deviations for luciferase activity (RLU) at 24 h (white bars) and 48 h (gray bars) after transfection.

replication level of each wild-type and chimeric replicon was evaluated by luciferase activity measurement after transient transfection of replicon RNA. No replication of any of the wild-type replicons (Fig. 4, JCH-1 wt, JCH-4 wt, H77c wt, and Con1 wt) or of any of the replicons with insertion of the JFH-1 NS3 helicase region (Fig. 4, JCH-1/N3H-JFH1, JCH-4/N3H-

JFH1, H77c/N3H-JFH1, and Con1/N3H-JFH1) was detected. However, genotype 2a replicons with insertion of the JFH-1 N5BX region increased their replication levels severalfold at 48 h (Fig. 4, JCH-1/N5BX-JFH1 and JCH-4/N5BX-JFH1). Furthermore, insertion of both the N3H and the N5BX regions increased the JCH-1 replication over 10-fold compared to that